

## Production of Itaconic Acid from *Jatropha curcas* Seed Cake by *Aspergillus terreus*

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### Abstract

Submerged substrate fermentation of *Jatropha* seed cake, a by-product of oil extraction from *Jatropha curcas* seed was carried out using *Aspergillus terreus* for the production of itaconic acid. The *Jatropha* seed cake was initially converted into fermentable sugars by dilute acid hydrolysis using 50% sulphuric acid. The rate of hydrolysis was 1.04 gL<sup>-1</sup>. The fermentation process was carried out at room temperature, agitation of 400 rpm and three physico-chemical parameters (pH, inoculum size and substrate concentration) were varied. Itaconic acid and glucose assays were carried out by spectrophotometry and Dinitrosalicylic acid methods respectively daily. Maximum yield of itaconic acid was 48.70 gL<sup>-1</sup> at 5 ml of inoculum size, 50% substrate concentration and pH 1.5. The residual glucose concentration increased for the first two days of fermentation after which it began to decrease as the itaconic acid concentration increased. The least concentration of itaconic acid observed was 6.00 gL<sup>-1</sup>, obtained after 24 hours of fermentation with 4 ml inoculum size, 50% substrate concentration and at pH 1.5. The findings of this work indicate that *Jatropha curcas* seed cake is a suitable substrate for itaconic acid production.

**Keywords:** acid hydrolysis, *Aspergillus terreus*, fermentation, itaconic acid, *Jatropha* seed cake, residual glucose

### Introduction

Itaconic acid, also known as methyl succinic acid, is an unsaturated di-carboxylic organic acid (Wilke and Vorlop, 2001) with the formula C<sub>5</sub>O<sub>4</sub>H<sub>4</sub>. Its primary application is in the polymer industry where it is employed as a co-monomer for the industrial synthesis of polyesters, plastics, and artificial glass; in the preparation of bioactive compounds in the agriculture, pharmacy, and medicine sectors; coatings, acrylic fibers, detergents, adhesives, thickeners, and binders, and a range of other products. It has a potential to be used as a substitute for acrylic or methyl methacrylate, both of which are derived from petrochemicals and other industrial products (Tate, 1981).

The first reported biological source of itaconic acid was *Aspergillus itaconicus*. Shortly thereafter, it was discovered that *A. terreus* also produced itaconic acid in higher quantities (Meena *et al.*, 2010) by *Ustilago maydis* (Chandragiri and Sastry, 2011a), various *Aspergillus* species and *Pseudozyma antarctica* (Levinson *et al.*, 2006).

Various agro-industrial wastes can be fermented for the production of itaconic acid. *Jatropha curcas* is also variously known as physic nut, purging nut, barbados nut, pig nut or fig nut (Encyclopedia of Fruits and Nuts, 2008), however *Jatropha* is both the genus and the most common name. The seeds contain anti-nutrients such as phorbol esters, trypsin inhibitors, lectins and phytate (Makkar *et al.*, 2008), thus rendering the seed and its products inedible.

The extraction of oil from *Jatropha* oilseeds and its conversion into biodiesel involves many complex processes in which a press-cake is a major byproduct. This cake is dark brown in color and contains carbohydrates, fibers and remaining oil and may be used as organic fertilizer because of the high content of nitrogen.

*Jatropha* is gaining increasing importance as an emerging source of biodiesel and this is leading to a rapid accumulation of the cake. Despite being nutrient-rich, *Jatropha* cake is toxic and cannot be consumed by humans or animals so it is cheap and readily available. Itaconic acid is currently expensive, so alternative or cheaper substrates may make the production process more profitable.

The process of obtaining itaconic acid from *Jatropha* cake like other plant biomass wastes involves three important steps- mechanical processing and pretreatment of the material to expose the cellulose and hemi-cellulose components; enzymatic or chemical hydrolysis of the pretreated material to produce reducing sugars such as glucose; and finally fermentation of the hydrolyzed material by a suitable microorganism to produce the desired acid (Zheng *et al.*, 2009).

The objectives of this work are to determine the suitability of *Jatropha curcas* seed cake as a cellulosic substrate for the fermentative production of itaconic acid, to determine the ability of *Aspergillus terreus* to utilize *Jatropha curcas* seed cake as substrate for itaconic acid production and lastly, to determine the optimum levels of parameters

such as substrate concentration, pH and inoculum size for itaconic acid production.

## Materials and methods

### *Preparation of jatropha seed cake (JSC)*

*Jatropha curcas* seeds were collected from Iwo, Osun state, and some markets in Kwara state, Nigeria. The cakes were initially crushed to 0.5-0.6 inches and soaked in water overnight to remove dirt and excess oil. The oil slowly left the cake to float on the water surface. The cakes were then separated from the dirty water using a sieve and dried in air (Mohit *et al.*, 2011).

### *Collection of microorganisms*

*Aspergillus terreus* was obtained from the Federal Institute of Industrial Research Oshodi (FIRO), Lagos state, Nigeria. The culture was maintained on potato dextrose agar (PDA) slants and kept at temperature of about 4°C prior to use.

### *Sucrose broth pre-culture medium*

Nutrient broth medium was prepared according to the manufacturer's specifications and was supplemented with 0.5 g sucrose. The broth was then sterilized in an autoclave at 121°C for 15 minutes at 15 psi. The sucrose broth was then inoculated with a loop full of *Aspergillus terreus* (Mohit, 2011; Rao, 2007).

### *Pre-treatment*

The size of the oil cakes was mechanically reduced using a grinder and a fine powder of 4.0 mesh size was obtained. The powder was dried in an oven at 45°C for 2 hours to completely remove the moisture. Equal volume of 0.5% H<sub>2</sub>SO<sub>4</sub> was added and the mixture was autoclaved at 121°C for 15 minutes at 15 psi. The mixture was again dried with warm air at 30-35°C (Mohit, 2011). Samples were collected from the pre-treated material for analysis.

### *Hydrolysis*

The raw materials were hydrolyzed by dilute acid hydrolysis method. Twice the volume of 5% H<sub>2</sub>SO<sub>4</sub> was added to the pre-treated material and mixed thoroughly. The mixture was poured in glass bottles and sealed to prevent vaporization of acid due to heat. The mixture was kept at a high temperature of 55°C for three days and was mixed regularly to prevent precipitation. Samples were collected from the hydrolyzed material daily for relevant analyses (Mohit, 2011).

### *Fermentation medium*

Specific quantities of *Jatropha* seed cake were mixed in 100ml of distilled water (Bresslen and Braun, 2000), 3 g NaNO<sub>3</sub>, 1 g K<sub>2</sub>HPO<sub>4</sub>, 0.8 g MgSO<sub>4</sub>, 0.01 g FeSO<sub>4</sub>, 0.5 g KCl, were added to the medium. The hydrolyzed medium was dispensed in bottles and sterilized in an autoclave at

121°C for 15 minutes at 15 psi. It was then inoculated with specific quantities of the spore suspensions, closed and incubated at room temperature on a rotary shaker at 400 rpm. Different parameters like inoculum size, substrate concentrations and pH were varied. The fermentation was continued for 15 days and samples were analyzed daily for residual glucose and itaconic acid (Mohit, 2011; Rao, 2007).

### *Variation of fermentation parameters*

#### *-pH*

The pH of the solution was adjusted to desired value by the addition of nitric acid and sodium hydroxide to pH values 2.0, 2.5, 3.0, 3.5 and 4.0. Other parameters were kept constant at 50% JSC concentration and inoculum size of 5%.

#### *-Substrate concentration*

This was varied at 10, 25, 40 and 50% of JSC in sterile distilled water. The pH was held at 1.55 and 5% inoculum size.

#### *-Inoculum size*

Spore suspension containing about  $9.3 \times 10^6$  spores per ml suspension were prepared according to the method of Pertruccioli *et al.* (1989) and used as inoculum for the fermentation process. This was varied from 1-5 ml to determine the effect of inoculum size on fermentative production of itaconic acid. The pH and substrate concentration were held at 1.55 and 50 g respectively.

### *Analytical methods and calculations*

Samples were collected daily. Glucose was estimated by Dinitrosalicylic acid (DNS) method and absorbance measured at 575 nm (Miller, 1959). The corresponding concentrations were obtained from a standard graph drawn from glucose standards. In this way, sugar estimation was done in the oilseed cakes before and after pretreatment-hydrolysis stage. Rate of hydrolysis was calculated using the formular of Arthe *et al.* (2008) and was used for calculating these two parameters:

$$\text{Rate of hydrolysis} = \frac{\text{Glucose}_t - \text{Glucose}_{t_0}}{t - t_0}$$

Where Glucose<sub>t</sub> is the concentration of glucose after time t, and Glucose<sub>t<sub>0</sub></sub> is the concentration of glucose before hydrolysis. t and t<sub>0</sub> are the final and initial time in hours respectively.

The itaconic acid produced was estimated by spectrophotometry (Meena *et al.*, 2010), and the absorbance was read at 385 nm, and again at 20 minutes to ascertain the reaction had gone to completion. The itaconic acid concentrations were then determined from standard graphs.

The data obtained after analysis were statistically analyzed by one way ANOVA. The experiments were dupli-

cated and the means were separated by Duncan's Multiple Range Test.

## Results and discussion

### Hydrolysis of JSC

The glucose content of the un-hydrolyzed and hydrolyzed JSC were 0.52 mg/g and 18.60 mg/g respectively. The rate of hydrolysis was  $1.04 \text{ g}^{-1}\text{kg}^{-1}\text{hr}$ . There was a significant increase ( $p < 0.05$ ) in the reducing sugar content of unhydrolyzed and hydrolyzed JSC and was estimated to be 0.014mg/ml and 0.089mg/g of the sample respectively. This is similar to the result obtained by Mohit *et al.* (2011) who observed that high volume of glucose was produced in the medium containing cellulose after three days hydrolysis with 5% sulfuric acid.

The highest quantity of itaconic acid, 48.70 g/L, was produced at pH 1.55, inoculum size 5 ml, substrate concentration of 50 g in 100 ml and aeration rate of 400rpm, at room temperature and this was obtained on the 9<sup>th</sup> day of fermentation. This figure compares favourably with the values such as  $60 \text{ gL}^{-1}$  obtained by Yahiro *et al.* (1997)  $20 \text{ gL}^{-1}$  obtained by Klement *et al.* (2012),  $24.45 \text{ gL}^{-1}$  by Rao *et al.* (2007) and  $97.14 \text{ gL}^{-1}$  by Chandragiri and Sastry (2011a). The lowest quantity of the acid was 6.00g/L produced after 24 hours of fermentation with 4 ml inoculum size.

### Effect of pH on fermentation

pH is one of the most important parameters that affect the production of itaconic acid by fermentation. Fermentable medium with adjusted pH of 2, 2.5, 3.5 and 4 were observed over a period of 15 days and maximum yield was obtained at all pH levels on Day 10 of fermentation with pH 4 yielding the highest itaconic acid concentration of  $38.70 \text{ gL}^{-1}$  (Tab. 1). This is close to the findings of Rao *et al.* (2007) and Chandragiri and Sastry (2011b) who reported the highest production of itaconic acid at pH 3.5 and 3 respectively. Production began to decline from Day 11 with the lowest itaconic acid production of  $8.50 \text{ gL}^{-1}$  obtained at day 14 as a result of the exhaustion of nutrient in the medium indicated by the low sugar in the medium (Fig. 1). Both the internal and environment's proton concentra-

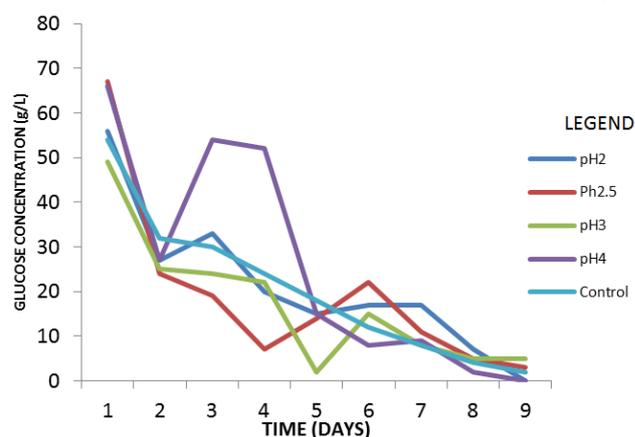


Fig. 1. Residual glucose in the fermentation at different pH conditions play a significant role on the growth and metabolism of a microorganism (Chandragiri and Sastry, 2011b); as a result microorganisms have evolved mechanisms to maintain relatively constant intracellular pH values, regardless of the pH of the external environment. However, when pH differs from what is optimal for any particular species, there will be an increase in maintenance energy required to maintain optimal intracellular pH and this may adversely affect the desired metabolite's production reflected in this research by declining yields of itaconic acid. This is similar to the report of Mohit *et al.* (2011) that the concentration of glucose broth was estimated to be around 9.75 mg/ml in the hydrolyzed Jatropha press cake which was reduced to 5.85 mg/ml at the end of fermentation.

### Effect of inoculum size on fermentation

Inoculum size is a major factor that determines the course of fungal fermentation. Inoculum sizes of 1, 2, 3, 4 and 5 ml were used for the fermentation of JSC to determine the optimum inoculum size for itaconic acid production at 50% substrate concentration, pH 1.55, ambient temperatures and aeration of 400 rpm. Maximum itaconic acid was obtained with 5ml of spore suspension ( $48.70 \text{ gL}^{-1}$ ) on the Day 9 of fermentation as shown in Fig. 2, even though there was no statistically significant difference ( $p < 0.05$ ) from the values observed at 2, 3, and 4ml of spore suspensions. This is similar to the findings of Chandragiri and Sastry (2011b), who observed that maximum produc-

Tab. 1. Itaconic acid production at different pH of fermentation

pH	Itaconic acid production (g/L) at different incubation time								
	Day 2	Day3	Day 4	Day 7	Day 8	Day 9	Day 10	Day 13	Day14
pH2.0	12.20±0.351 <sup>b</sup>	13.00±0.52 <sup>c</sup>	19.50±3.92 <sup>c</sup>	20.00±1.36 <sup>bc</sup>	24.00±2.04 <sup>a</sup>	24.50±0.11 <sup>a</sup>	37.00±0.75 <sup>d</sup>	24.00±0.37 <sup>d</sup>	8.50±0.22 <sup>a</sup>
pH2.5	12.00±0.59 <sup>b</sup>	16.00±0.83 <sup>c</sup>	23.50±0.82 <sup>d</sup>	14.50±1.68 <sup>a</sup>	28.70±0.39 <sup>b</sup>	29.50±0.41 <sup>b</sup>	31.20±0.182 <sup>c</sup>	19.20±0.816 <sup>c</sup>	11.20±0.365 <sup>cd</sup>
pH3	11.50±2.16 <sup>b</sup>	14.50±0.91 <sup>d</sup>	19.20±0.14 <sup>c</sup>	21.70±4.20 <sup>c</sup>	24.10±0.28 <sup>a</sup>	24.50±0.41 <sup>a</sup>	22.00±1.41 <sup>b</sup>	11.70±0.16 <sup>a</sup>	10.00±0.93 <sup>ab</sup>
pH4	19.10±0.08 <sup>a</sup>	16.60±0.55 <sup>a</sup>	13.00±0.18 <sup>a</sup>	19.70±0.22 <sup>b</sup>	23.50±0.82 <sup>a</sup>	24.50±0.42 <sup>a</sup>	38.70±0.16 <sup>c</sup>	20.00±0.99 <sup>c</sup>	11.50±0.14 <sup>d</sup>
Control	9.50±0.21 <sup>a</sup>	11.70±0.16 <sup>b</sup>	14.50±2.45 <sup>b</sup>	18.20±1.41 <sup>b</sup>	24.50±0.34 <sup>a</sup>	28.00±0.26 <sup>b</sup>	19.00±0.36 <sup>a</sup>	15.20±0.28 <sup>b</sup>	10.20±0.44 <sup>bc</sup>

Other conditions were kept constant: 5 ml Inoculum size and 50 g of substrate concentration. The control is at pH 1.55

The values are mean of four replicates ± standard deviation. Values with different superscripts within the same column show significant difference ( $p < 0.05$ )

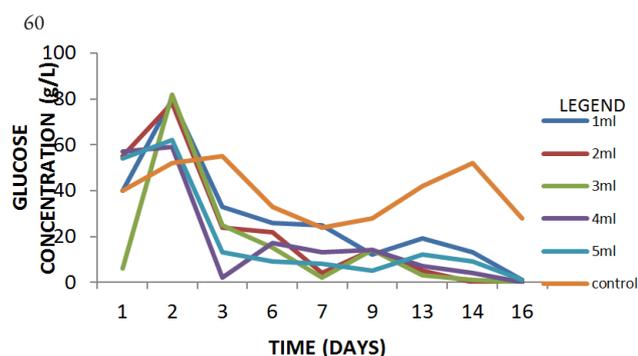


Fig. 2. Residual glucose during the fermentation with different inoculum sizes

tion of itaconic acid was obtained with 5 ml of inoculum size of *Ustilago maydis*. This observation implies more of the organism produces more itaconic acid and this also explains the reduction in reducing sugar ( $0.005 \text{ gL}^{-1}$ ) during fermentation with this inoculum size (Tab. 2). The lowest production of itaconic acid was 6.00 mg/ml on 4 ml inoculum after 24 hours of fermentation.

It was also observed that residual glucose increased in the first two days before the concentration began to decline. This observation is in line with what Mohit *et al.* (2011) observed during production of ethanol by *Saccharomyces cerevisiae* using *Jatropha curcas* seed cake that the rate of consumption of glucose is lower in the first 4 days of fermentation, but picks up in later days. This is probably because the hydrolysis of the JSC is proceeding with time, thus liberating more sugars from the cake because the organism was undergoing a lag phase while adjusting to the new environment of the fermentation medium. This also explains the low itaconic acid quantities observed on Day 1 during the various experiments and the rapid increase on Day 2 (Fig. 1-3).

Tab. 2. Effect of inoculum size on itaconic acid production

Inoculum Size	Itaconic acid production (g/L)								
	Day 1	Day2	Day3	Day6	Day7	Day9	Day13	Day14	Day15
1 ml	8.00±0.18 <sup>b</sup>	9.50±0.26 <sup>a</sup>	10.20±0.08 <sup>b</sup>	12.50±0.91 <sup>b</sup>	39.00±0.43 <sup>c</sup>	42.00±1.47 <sup>b</sup>	32.00±0.91 <sup>b</sup>	35.50±2.25 <sup>c</sup>	32.20±0.18 <sup>d</sup>
2 ml	9.50±1.25 <sup>c</sup>	10.50±0.41 <sup>b</sup>	19.70±0.16 <sup>d</sup>	20.70±0.18 <sup>c</sup>	33.50±0.26 <sup>c</sup>	47.00±1.10 <sup>c</sup>	35.10±0.18 <sup>bc</sup>	26.60±0.37 <sup>b</sup>	12.00±3.39 <sup>b</sup>
3 ml	10.70±0.18 <sup>c</sup>	12.20±0.50 <sup>c</sup>	12.20±0.32 <sup>c</sup>	10.20±0.14 <sup>c</sup>	32.00±1.47 <sup>b</sup>	47.70±0.18 <sup>cd</sup>	37.20±0.14 <sup>c</sup>	37.00±0.91 <sup>d</sup>	14.50±2.08 <sup>b</sup>
4 ml	6.00±0.832 <sup>a</sup>	13.70±0.83 <sup>d</sup>	10.50±0.03 <sup>b</sup>	21.20±0.18 <sup>a</sup>	35.10±0.14 <sup>d</sup>	48.00±0.18 <sup>cd</sup>	36.00±0.41 <sup>c</sup>	33.70±0.91 <sup>d</sup>	26.10±0.91 <sup>b</sup>
5 ml	17.20±0.73 <sup>c</sup>	27.00±0.43 <sup>c</sup>	21.10±0.03 <sup>c</sup>	23.50±0.44 <sup>d</sup>	31.70±0.36 <sup>b</sup>	48.70±0.41 <sup>d</sup>	35.00±0.48 <sup>bc</sup>	38.60±0.37 <sup>d</sup>	24.80±0.37 <sup>c</sup>
Control	6.00±0.83 <sup>a</sup>	8.50±0.29 <sup>a</sup>	6.00±0.83 <sup>a</sup>	8.50±0.09 <sup>a</sup>	9.70±0.29 <sup>a</sup>	8.00±0.18 <sup>a</sup>	10.80±0.47 <sup>a</sup>	9.50±0.26 <sup>a</sup>	9.30±0.41 <sup>a</sup>

Other conditions were kept constant: pH 1.55 and 50g of substrate concentration. The values are mean of four replicates ± standard deviation. Values with different superscripts within the same column show significant difference ( $p < 0.05$ )

Tab. 3. Itaconic acid estimation at different substrate concentration

Substrate concentration	Itaconic acid production (g/L)					
	Day 1	Day2	Day4	Day7	Day9	Day10
10%	8.000±0.036 <sup>b</sup>	10.00±0.056 <sup>a</sup>	15.001±0.018 <sup>b</sup>	17.00±0.083 <sup>a</sup>	9.000±0.028 <sup>a</sup>	7.100±0.018 <sup>a</sup>
25%	15.10±0.018 <sup>a</sup>	17.00±0.036 <sup>b</sup>	8.100±0.014 <sup>a</sup>	25.20±0.052 <sup>b</sup>	20.10±0.018 <sup>b</sup>	13.0±0.018 <sup>b</sup>
40%	18.50±0.082 <sup>b</sup>	20.00±0.036 <sup>c</sup>	20.50±0.030 <sup>d</sup>	31.70±0.018 <sup>c</sup>	30.00±0.044 <sup>c</sup>	16.60±0.052 <sup>c</sup>
50%	20.60±0.036 <sup>c</sup>	21.00±0.055 <sup>c</sup>	19.70±0.016 <sup>c</sup>	26.70±0.026 <sup>a</sup>	32.00±0.088 <sup>d</sup>	18.00±0.036 <sup>d</sup>

Other conditions were kept constant: pH 1.55 and 5 ml inoculum size. The values are mean of four replicates ± standard deviation. Values with different superscripts within the same column show significant difference ( $p < 0.05$ )

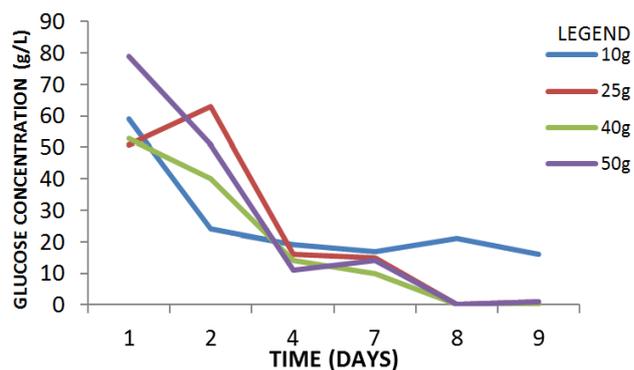


Fig. 3. Residual glucose in the fermentation at different substrate concs

#### Effect of varying substrate concentration

A comparative account of production of itaconic acid using *Jatropha curcas* seed cake at different concentrations i.e. 10%, 25%, 40% and 50% using *A. terreus* was also studied. The maximum production of itaconic acid obtained was 31.70 g/L at Day 7 of fermentation with 40% JSC (Tab. 3).

This result is similar to that of Chandragiri and Sastry (2011b) who obtained maximum production of itaconic acid on 35% concentration of pure glucose. This value was significantly higher than that of other concentrations on the same day. The lowest production of itaconic acid is 8.00 g/L with 10 g substrate concentration which is to be expected because of the low JSC concentration and also, it was on the first day of fermentation where there may be a lag phase. It was observed that with increase in itaconic acid production, there was a corresponding decrease in glucose concentration (Fig. 3).

## Conclusions

Jatropha seed cake (JSC) has been shown to be suitable for the fermentative production of itaconic acid. This work shows that the substrate concentration, incubation time, inoculum size and pH all affect the production of itaconic acid on Jatropha seed cake and the optimized physico-chemical parameters for fermentation of Jatropha Seed Cake for the production of itaconic acid are: pH 4 inoculum size of 5 ml and JSC concentration of 40%. As the cake which is a waste byproduct of the biodiesel production process accumulates over time, it can be utilized in a bid for value addition, as a good raw material for itaconic acid production and at the same time, solve the problem of safe disposal of the waste.

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